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14. ABSTRACT

The plasminogen cascade of serine proteases has been affiliated in the mammary gland with both development and tumorigenesis. We have found that the dominant plasminogen activator during mammary gland stromal involution is plasma kallikrein (PKal), and that active PKal appears in connective tissue-type mast cells in the mammary stromal during different phases of development. Thus, to determine the role of PKal in mammary gland involution, a prekallikrein-deficient mouse is being produced that will be analyzed for developmental defects as well as defects in mammary gland tumor metastasis. After unanticipated difficulties in the cloning of a knockout construct, correctly targeted embryonic stem cell lines have been established that will be used to make the PKal knockout mouse. Additionally, examination of the extra hepatic expression of PKal has shown that PKal message is present in the mammary gland, and that increased expression levels correlate to periods of stromal remodeling.

15. SUBJECT TERMS
mammary gland, serine protease, plasma kallikrein, mast cell, mouse model, involution,adipocyte, extracellular matrix, plasminogen

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INTRODUCTION

The plasminogen cascade of serine proteases has been affiliated in the mammary gland with both development and tumorigenesis. The ultimate effector in this cascade, plasminogen (active form: plasmin), is managed by an intricate cascade of plasminogen activators and protease inhibitors. Plasminogen activators and inhibitors are strongly associated with poor prognosis in a variety of human tumors, including breast cancer. [1] Furthermore, plasminogen-deficient mice yield significantly fewer metastases in a viral oncogene-induced model of breast cancer[2, 3] and exhibit significant defects in lactational competence and post-lactational mammary gland involution.[4] This study focuses on the role of a plasminogen activator in murine mammary stromal development and metastasis. This lab has demonstrated that the dominant plasminogen activator for mammary stromal involution is plasma kallikrein (PKal)[5] and that active PKal appears in connective tissue-type mast cells in the stroma and surrounding the blood vessels of the murine mammary gland. This project aims to study the plausible relationship between mast cell activation, PKal release, and plasmin conversion in the mammary gland by examining the role of PKal in mammary gland involution and metastasis. This report details the work accomplished during the first year of my predoctoral traineeship award.

BODY

Task 1. The generation of a prekallikrein-deficient mouse is the crucial first step towards determining the effects of the loss of PKal on mammary gland development and tumorigenesis. Prekallikrein consists of two domains: a binding domain, and a trypsin-like serine protease domain (Figure 1). Its binding domain, consisting of four apple (PAN) domains, is thought to mediate interactions between prekallikrein and plasma-borne PKal activators such as Factor XII.[6, 7] PKal's serine protease domain is highly conserved amongst other proteases in the plasminogen cascade as well as other members of the large trypsin-like protease family. Previous attempts in this laboratory to generate a prekallikrein-deficient mouse targeted the catalytic residues of the protease domain. The knockout construct for this strategy failed to generate a correctly targeted embryonic stem (ES) cell clone, so a new strategy was employed to target the prekallikrein start codon.

At the time of the beginning of this award, a knockout construct was half-completed that would insert the gene encoding green fluorescent protein (GFP) behind the endogenous start codon for prekallikrein. Due to difficulties during the cloning process, the original strategy was modified: firstly, one of the homology arms was difficult to amplify by polymerase chain reaction (PCR); secondly, the drug resistance cassette that was originally used (Puromycin resistance) had to be exchanged for another selection cassette (Hygromycin resistance) to avoid recombination events between too-similar polyA sequences at the end of the GFP cassette and the Puromycin resistance cassette (Figure 2). The final knockout construct was completed in month 6 of the award.

Task 1a. Screening of embryonic stem (ES) cell colonies for a correctly targeted allele required screening of 144 cell lines generated by electroporation of the PKal knockout construct into E14 (SV129/Ola) ES cells. Screening by Southern blot hybridization using standard methods also proved difficult. Eight different probes were generated against regions 5' and 3' to the homology arms of the knockout construct, and all yielded inconsistent or weak signals with significant background, making screening of the candidate cell lines inconclusive. An alternate strategy using an internal probe consisting of the majority of the Hygromycin resistance coding sequence resulted in clean Southern blots with strong signal, so correct targeting had to be

determined using multiple independent restriction digestions of candidate DNA. Subsequent analysis resulted in the identification of three ES cell lines that are correctly targeted. A genotyping strategy using PCR is being developed at this time, and the three targeted clones are currently being analyzed for correct karyotype to ensure that transmission of the knockout allele will not be complicated by unanticipated chromosomal abnormalities.

The Statement of Work for this award did not allow for difficulties in the final cloning steps for the knockout construct, nor for in the ES cell screening, therefore the timeline for Task 1 has been significantly delayed. The locus for the plasma kallikrein gene has proven to be a difficult template for many standard molecular biology techniques such as PCR, cloning, and Southern hybridization. In fact, the annotation for the gene was removed from the Ensembl database (www.ensembl.org/mus_musculus) in the last three assemblies of the mouse genome. Communication with the Ensembl consortium has revealed that the PKal annotation (Klkb1) has been temporarily removed due to multiple sequencing difficulties in the locus. Comparison of the sequence used to create the PKal knockout construct employed in this project with other published sequence (NCBI and published bacterial artificial chromosome (BAC)) data for the prekallikrein locus confirms that use of this construct should not fail to produce a prekallikrein-deficient mouse, so *Task 1b*, the injection of blastocysts with correctly targeted ES cells will take place upon receipt of the karyotype analysis as discussed above.

Task 1c + 1d. Mouse breeding and the beginning of analysis on PKal-null animals has been delayed as explained above and will proceed as soon as mice are generated.

Task 2. Analysis of plasma kallikrein expression in the mouse mammary gland is necessary to confirm preliminary data suggesting extrahepatic expression of PKal in the mammary gland stroma and/or in connective tissue-type mast cells. If plasma kallikrein is produced outside of the liver in tissues that require its activity and is activated non-canonically apart from the contact activation system in blood vessels, then this would represent a novel pathway for the plasminogen cascade of protease activity. It has recently been shown that plasminogen is expressed by a wide range of tissues [8] therefore, it is not unreasonable to hypothesize that tissues in which plasmin activity is required would also have locally expressed plasminogen activators to better control the activation cascade.

Task 2a. Plasma kallikrein expression in the mouse mammary gland at different developmental time points (1 week, 3 weeks, 5 weeks, 15 days pregnant, 10 days lactating, and 4 days involuting) was assessed using real-time PCR. Mammary glands collected at 1 week of age did not yield sufficient quality RNA to produce consistent results; protein levels may prove to be a better measure in future experiments. Results shown in Figure 3 compare levels of prekallikrein message at 5 time points in mammary gland development. Prekallikrein message is present strongly during virgin development, when the mammary gland is undergoing active remodeling as the ductal epithelium expands and advances through the stromal fat pad. During pregnancy and lactation, when the stroma has largely been replaced by secretory alveoli and extensive ductal structures, prekallikrein message is markedly reduced. Prekallikrein message increases significantly during the program of mammary gland involution, when the secretory lactation structures apoptose and the mammary stromal compartment is replenished. These findings confirm that not only is PKal produced in the mammary gland, but that increased expression levels correlate to periods of stromal remodeling.

Task 2b. Using ecotin PKal, a macromolecular inhibitor of active PKal [9], we have shown that inhibition of PKal during mammary gland involution significantly inhibits adipocyte

replenishment and stromal remodeling (J. Lilla, unpublished data). To confirm that the target of this inhibitor *in vivo* is PKal, and to determine whether other factors are affected by ecotin PKal, I have collected mammary gland lysates from different stages of development to be analyzed for binding partners to ecotin PKal. Analysis has not yet occurred, as the experimental plan was dependent on the availability of prekallikrein-deficient mice generated from the first set of tasks (see above) to serve as an essential negative control for this set of experiments. Once the PKal knockout mice are available, their mammary glands will be harvested and added to the collection of tissues for ecotin PKal binding partner analysis.

KEY RESEARCH ACCOMPLISHMENTS

- The prekallikrein locus is resistant to standard molecular biology techniques.
- A construct to replace the first exon of the prekallikrein gene with GFP has been introduced into mouse ES cells and correctly targeted cell lines have been established.
- Prekallikrein message is present in the mammary gland, and increased levels correlate to phases of stromal remodeling.

REPORTABLE OUTCOMES

None to date.

CONCLUSION

Though unforeseeable delays to the generation of a prekallikrein-deficient mouse line have affected much of the work of this first year of the predoctoral award period, it is anticipated that significant progress will be made in the second year. To make up for these delays and increase productivity during the second year, in the last two months breeding and analysis of the mammary gland development of *W-sash* mice deficient in connective tissue-type mast cells [10] was begun ahead of schedule (Task 2d in the Statement of Work). Additionally, breedings to generate transgene-driven mammary cancer in the *W-sash* mouse line were recently begun as an inquiry into the role of mast cell activity in mammary gland tumorigeneis and metastasis.

Plasma kallikrein activity appears to be associated with the function of two major stromal cell types of the mammary gland: mast cells and adipocytes. This research aims to elucidate the role of PKal in mammary gland involution and metastasis. As many models of breast cancer highly implicate stromal signals as effectors or inhibitors of breast cancer progression, it is imperative to acquire a better understanding of the normal functioning of this protease to prepare for its possible characterization as a breast cancer indicator like other members of the plasminogen protease cascade, or as a target for drug therapies.

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APPENDICES

None.

SUPPORTING DATA

Figure 1. Domains of Plasma Kallikrein
Prekallikrein (active form: Plasma Kallikrein) consists of 4 apple (PAN) domains and a trypsin-like serine protease domain (catalytic residues H-D-S). Activation of the protease requires cleavage between the heavy and light chains by Factor XII.

Figure 2. Targeting construct to generate the PKal knockout mouse.
The endogenous start codon is located at the 3' end of the 5' homology arm. Upon proper recombination, the first exon is replaced with the coding sequence for GFP.

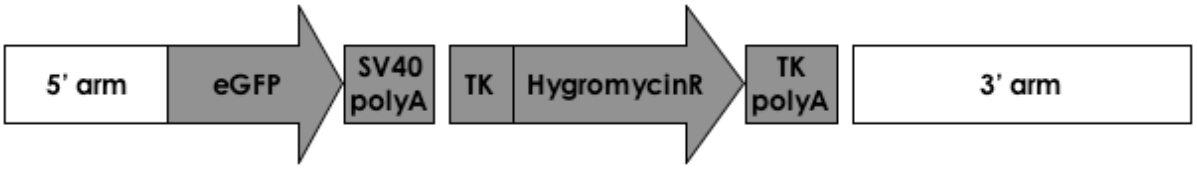

Figure 3. Real-time PCR analysis of plasma kallikrein expression in mammary gland RNA